

### **AMENDMENTS TO THE CLAIMS:**

This listing of claims will replace all prior versions and listings of claims in the application:

1. (Withdrawn) A sulfur atom-free enzyme protein comprising 18 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; and L-tryptophan.

2. (Withdrawn) The sulfur atom-free enzyme protein according to claim 1 which retains the activity of the original enzyme protein and has oxidation resistance, wherein L-cystein and L-methionine residues in enzyme proteins comprising 20 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; L-tryptophan; L-cystein; and L-methionine, are substituted with 18 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; and L-tryptophan.

3. (Withdrawn) The sulfur atom-free enzyme protein according to claim 2 wherein amino acid substitution is carried out by site-directed mutagenesis using synthetic DNA.

FINNEGAN  
HENDERSON  
FARABOW  
GARRETT &  
DUNNER LLP

1300 I Street, NW  
Washington, DC 20005  
202.408.4000  
Fax 202.408.4400  
www.finnegan.com

4. (Withdrawn) The sulfur atom-free enzyme protein according to any one of claims 1 to 3 wherein the enzyme activity is oxidation-reduction activity or hydrolysis activity.

5. (Withdrawn) The sulfur atom-free enzyme protein according to any one of claims 1 to 4, which retains the activity of dihydrofolate reductase and has oxidation resistance.

6. (Withdrawn) The sulfur atom-free enzyme protein according to any one of claims 1 to 4, which retains the activity of xylanase and has oxidation resistance.

7. (Currently amended) A method of producing a sulfur atom-free enzyme protein having activity greater than or equivalent to an original enzyme protein, wherein the sulfur atom-free enzyme protein is prepared by a combined mutation method comprising ~~the following steps:~~

(1) preparing a mutant gene by substituting an initiation codon encoding L-methionine in a DNA sequence encoding an enzyme protein ~~consisting of a total length of m amino acids, and~~ having an amino acid sequence comprising n number of sulfur atom-containing amino acids (~~sulfur-containing amino acids~~), wherein a position of a sulfur-containing amino acid on the sequence is  $A_i$  ( $i = 1$  to  $n$ ), by with codons encoding any of L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline ~~codon;[[,]]~~ expressing the ~~prepared~~ mutant gene in a host cell to obtain a mutant enzyme protein;[[,]] measuring enzyme activity of the ~~obtained~~ mutant enzyme protein;[[,]] and

selecting the mutant enzyme protein with the highest activity, thereby obtaining a substitution mutant enzyme protein having a substitution A1/MA1;

(2) preparing a mutant gene in which ~~codons~~ a codon encoding a sulfur-containing amino acid ~~at another sites~~ a position  $A_i$  ( $i = 2$  to  $n$ ) ~~are is~~ substituted with a codon ~~encoding another amino acids among the 18 types of amino acid according to claim 1,~~ any of L-alanine, L-aspartic acid, L-glutamic acid, L-phenylalanine, L-glycine, L-histidine, L-isoleucine, L-lysine, L-leucine, L-asparagine, L-proline, L-glutamine, L-arginine, L-serine, L-threonine, L-valine, L-tyrosine, and L-tryptophan, for a maximum of 18 different substitutions at any  $A_i$  ~~expressing the prepared mutant gene in a host cell to obtain a mutant enzyme protein; measuring enzyme activity of the obtained mutant enzyme protein; and selecting p-number of mutant enzyme proteins having enzyme activity, thereby obtaining substitution mutants~~  $A_i/B_{ij}$  ( $j = 1$  to  $p$ );

(3) ~~selecting a maximum of 3 substitution mutants~~ a maximum of three mutant enzyme proteins having the highest activity, thereby obtaining mutant enzyme proteins having substitutions  $A_i/B_{i1}$ ,  $A_i/B_{i2}$ , and  $A_i/B_{i3}$ , wherein activity decreases in order ~~in order~~  $A_i/B_{i1} > A_i/B_{i2} > A_i/B_{i3} > \dots > A_i/B_{ip}$ ; and

(3) repeating (2) for all  $A_i$ ; and

(4) ~~selecting substitution mutants having activity in respect of sulfur-containing amino acids at all sites  $A_i$  ( $i = 2$  to  $n$ ) in the same manner as (2) and (3) above, preparing a maximum of  $3 \times (n - i)$  mutants being all combinations of these mutants with the mutant producing a sulfur atom-free enzyme protein comprising any combination of the substitutions in (2) and (3) with the~~

substitution of A1/MA1 in (1), wherein a substitution occurs at all  $A_i$ ;

measuring the enzyme activity ~~thereof~~ of the sulfur atom-free enzyme protein;

and ~~obtaining~~ selecting a mutant sulfur atom-free enzyme protein having activity greater than or equivalent to that of the original enzyme protein.

8. (Currently Amended) A method of producing a sulfur atom-free enzyme protein having activity greater than or equivalent to an original enzyme protein, wherein the sulfur atom-free enzyme protein is prepared by a stepwise mutation method comprising the following steps:

(1) preparing a mutant gene by substituting an initiation codon encoding L-methionine in a DNA sequence encoding an enzyme protein ~~consisting of a total length of m amino acids, and~~ having an amino acid sequence comprising n number of sulfur atom-containing amino acids (~~sulfur-containing amino acids~~), wherein a position of a sulfur-containing amino acid on the sequence is  $A_i$  ( $i = 1$  to  $n$ ), ~~by~~ with codons encoding any of L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline ~~codon;~~ expressing the prepared mutant gene in a host cell to obtain a mutant enzyme protein; measuring enzyme activity of the ~~obtained~~ mutant enzyme protein; and selecting the mutant enzyme protein with the highest activity, thereby obtaining a ~~substitution~~ an A1/MA1 mutant A1/MA1;

(2) preparing a mutant gene in which ~~codons~~ a codon encoding a sulfur-containing amino acid  $[[s]]$  at  $A_2$  of the A1/MA1 mutant of (1) is substituted with a codon encoding ~~another amino acid~~ any of L-alanine, L-aspartic acid, L-glutamic acid, L-phenylalanine, L-glycine, L-histidine, L-

isoleucine, L-lysine, L-leucine, L-asparagine, L-proline, L-glutamine, L-arginine, L-serine, L-threonine, L-valine, L-tyrosine, and L-tryptophan, for a maximum of 18 different substitutions at A2; being one of the 18 types of amino acid  
according to claim 1, expressing the prepared mutant gene in a host cell to  
obtain a double mutant;[[,]] measuring enzyme activity of the obtained double  
mutant enzyme protein;<sub>i</sub> and selecting a maximum of 3 triple double mutants with  
the highest activity;

(3) preparing a mutant gene in which a codon encoding a sulfur-containing amino acid at each A3 of the obtained double mutants is substituted with a codon encoding another amino acid any of L-alanine, L-aspartic acid, L-glutamic acid, L-phenylalanine, L-glycine, L-histidine, L-isoleucine, L-lysine, L-leucine, L-asparagine, L-proline, L-glutamine, L-arginine, L-serine, L-threonine, L-valine, L-tyrosine, and L-tryptophan, for a maximum of 18 different  
substitutions at A3; being one of the 18 types of amino acid according to claim 1,  
expressing the prepared mutant gene in a host cell to obtain a triple mutant;[[,]]  
measuring enzyme activity of the obtained triple mutant enzyme protein;<sub>i</sub> and  
selecting a maximum of [[3]] three triple mutants with the highest activity; and

(4) repeating the stepwise substitution of codons encoding sulfur  
containing amino acids at each remaining A<sub>i</sub> (i = 4 to n) in the same manner as  
described above in (2) and (3), wherein positions A<sub>i</sub>-A<sub>n</sub> are substituted in any  
order; preparing a quadruple mutant, ..., multiple number of n mutant, inspecting  
measuring enzyme activity of last multiple number of n mutant, a sulfur atom-free  
enzyme protein obtained upon substitution of A<sub>n</sub>; and preparing selecting a

~~mutant sulfur atom-free enzyme protein having activity greater than or equivalent to that of the original enzyme protein.~~

9. The process for producing a sulfur atom-free enzyme protein prepared by the stepwise mutation method according to of claim 8 wherein A1-An are substituted in order of position on the amino acid sequence ~~the order of stepwise mutation sites is according to any one of (n! types) permutations and combinations of A1, A2, ..., An.~~

10. (Currently amended) A process for producing a sulfur atom-free enzyme protein using a combination of a combined mutation method and a stepwise mutation method, wherein the method comprises:, ~~in an enzyme protein consisting of a total length of m amino acids and comprising n number of sulfur-containing amino acids, in which a site of a sulfur-containing amino acid on the sequence is  $A_i$  ( $i = 1$  to  $n$ ), a process according to claim 7 is adopted at k number of sites and a process according to claim 9 is adopted at remaining  $n - k$  number of sites.~~

(1) preparing a mutant gene by substituting an initiation codon encoding L-methionine in a DNA sequence encoding an enzyme protein having an amino acid sequence comprising n number of sulfur atom-containing amino acids, wherein a position of a sulfur-containing amino acid to be substituted is designated as  $A_i$  ( $i = 1$  to  $k$ ,  $k \leq n$ ), with codons encoding any of L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline; expressing the mutant gene in a host cell to obtain a mutant enzyme protein; measuring enzyme activity of the mutant enzyme protein; and selecting the protein with the highest activity, thereby obtaining a mutant enzyme protein having a substitution  $A1/MA1$ ;

(2) preparing a mutant gene in which a codon encoding a sulfur-containing amino acid at a position  $A_i$  ( $i = 2$  to  $k$ ) is substituted with a codon encoding any of L-alanine, L-aspartic acid, L-glutamic acid, L-phenylalanine, L-glycine, L-histidine, L-isoleucine, L-lysine, L-leucine, L-asparagine, L-proline, L-glutamine, L-arginine, L-serine, L-threonine, L-valine, L-tyrosine, and L-tryptophan, for a maximum of 18 different substitutions at any  $A_i$ ; expressing the mutant gene in a host cell to obtain a mutant enzyme protein; measuring enzyme activity of the mutant enzyme protein; and selecting a maximum of three mutant enzyme proteins having the highest activity, thereby obtaining mutant enzyme proteins having substitutions  $A_i/Bi1$ ,  $A_i/Bi2$ , and  $A_i/Bi3$ , wherein activity decreases in order  $A_i/Bi1 > A_i/Bi2 > A_i/Bi3$ ; and

(3) repeating (2) for all  $A_i$ ;

(4) producing a mutant enzyme protein comprising any combination of substitutions as in (1), (2), and (3), wherein a substitution occurs at all  $A_i$ ; measuring enzyme activity of the mutant enzyme protein; and selecting a maximum of three mutant enzyme proteins having the highest activity; and

(5) producing a sulfur atom-free enzyme protein by subjecting the mutant enzyme proteins selected in (4) to stepwise substitution of the remaining  $n - k$  number of sulfur-containing amino acids as in claim 8; measuring the enzyme activity of the sulfur atom-free enzyme protein; and selecting a sulfur atom-free enzyme protein having activity greater than or equivalent to that of the original enzyme protein.

FINNEGAN  
HENDERSON  
FARABOW  
GARRETT &  
DUNNER LLP

1300 I Street, NW  
Washington, DC 20005  
202.408.4000  
Fax 202.408.4400  
www.finnegan.com